Monitoring Metal Concentrations in Tissues and Single Cells Using Ultramicrosensors

Tadeusz Malinski, Saul Grunfeld, Ziad Taha, and Paul Tomboulian

Department of Chemistry, Oakland University, Rochester, Michigan

Intercellular and extracellular metal concentrations were measured using carbon fiber ultramicrosensors plated with mercury or with polymeric porphyrinic p-type semiconductors. Concentrations of unbound nickel and lead ions were studied within individual BC3H-1 myocytes, and H4-11-C3 rat hepatoma cells. Unbound ions are predominantly solvated inorganic ions not coordinated to biological cellular components. Fabrication of ultramicrosensors appropriate for the cells under investigation is described, including procedures for sharpening and waxing the microsensors in order to control the shape, area, and dimensions of the electroactive surface. Metal ion movement through cell membranes and intracellular ion diffusion in aorta tissue were studied. — Environ Health Perspect 102(Suppl 3):147–151 (1994).

Key words: accumulation, nickel, lead, single cells, microsensors.

Introduction

Of the 30 elements believed to be essential for most animal organisms, nine are transition metals, including the three biologically active trace metals Fe, Zn, and Cu. The remainder, i.e., V, Cr, Mn, Mo, Co, and Ni, are considered ultratrace since less than 10 mg are required or found in the adult human (<130 ppb) (*1*). These ultratrace elements normally occur and function in biologic fluids and tissues at low concentrations (10^{-6} to 10^{-9} M) and are maintained within narrow optimal ranges, thus defining states of deficiency and toxicity. Some other ultratrace elements, such as Pb, As, or Cd, exert toxic effects at similar low concentrations (*2*). Total metal concentrations in organs and tissues have been determined using atomic spectroscopy and X-ray fluorescence (*3,4*). Accumulation of higher concentrations of metals in tissues can be monitored using autoradiographic or liquid scintillation counting techniques. However, neither spectroscopic nor radiochemical techniques can differentiate between metal bound by proteins or adsorbed on cellular membranes, and unbound metal in intracellular fluids. Studies of mechanisms of accumulation, retention, and clearance may be expedited by data obtained through studies of single cells rather than whole body organ data (*5*). Cellular comparisons will allow differentiation of effects due to the nature of the cell, such as membrane permeability and cell volume, rather than the efficacy of physiological defense mechanisms.

This paper describes a methodology for measuring concentrations of unbound metals in single biologic cells. The methodology is demonstrated for nickel and lead, but a similar procedure can be applied for several other trace metals in biological systems.

Nickel is known to produce deleterious biological effects including carcinogenicity as well as embryonic and fetal toxicity. A recent review (*6*) of nickel toxicity addressed three broad categories: systemic toxicity, molecular toxicology, carcinogenicity and genotoxicity. In addition to genotoxicity and carcinogenicity, another review (*7*) discussed aspects of exposure and dosage, including occurrence, transport and alterations, bioavailability, accumulation, metabolism, speciation, and routes of exposure.

Lead is extremely poisonous because it can bind in much the same places as Zn^{2+} and Ca^{2+} in enzyme systems, producing neurotoxicity, peripheral neuropathy, hypertension, and a variety of other disorders (*8*).

Materials and Methods

Reagents and Materials

Tetrakis(3-methoxy-4-hydroxyphenyl)-nickel porphyrin (TMHPPNi) was synthesized according to a procedure described previously (*9*). Celion 650-300 carbon fibers with a 6 μm diameter were obtained from BASF (Troy, MI). Waxes used for electrode coating were beeswax with 10% rosin. All acids and bases were of Suprapure grade (Ultrex, J.T. Baker, Philipsburg, NJ). Standard solutions of Ni(II) and Pb(II) cations were prepared from 1000 μg/ml ICP reference stock standards (Spec Industries, North Haven, CT). BC3H-1 myocytes were plated at an initial density of $1.5 \times 10^6 - 2 \times 10^6$ cells/cm^2 (Dulbecco's modified Eagle medium and 15% controlled process serum replacement, Sigma, St. Louis, MO) onto glass plates (area ca. 1.0 cm^2). H4-11-C3 rat hepatoma cells were grown on glass plates with Dulbecco's modified Eagle medium containing L-glutamine (4 mM) and 15% controlled process serum replacement (Sigma, Chicago, IL). Cells on glass plates were incubated at 37°C under 5% carbon dioxide. Porcine aorta was placed in a petri dish containing DMEM cell culture media (Fisher). The aorta was cut into rings (3–5 mm thickness), each placed into a petri dish containing fresh culture medium. A differential pulse voltammetry (DPV) and anodic stripping voltammetry (ASV) were used to monitor the analytical signal, due to oxidation of Ni(II) to Ni(III) in the porphyrinic polymeric film, or oxidation of Pb(0) accumulated in mercury to Pb(II).

Apparatus

A three-electrode system in a quartz cell was used for the porphyrin film formation by electrode deposition, and for Ni(II) and Pb(II) determination by DPV and ASV, using a sharpened carbon fiber as the working electrode. The auxiliary and reference electrodes were platinum wire and standard calomel electrode (SCE), respectively.

A PAR model 264A voltammetric analyzer with PAR model 181 current-sensitive preamplifier (Princeton, NJ) was used for DPV and ASV. Current-voltage curves were recorded with PAR model 9002A X-Y recorder.

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Address all correspondence to Dr. Tadeusz Malinski, Department of Chemistry, Oakland University, Rochester, MI 48309-4411. Telephone (313) 370-2339. Fax (313) 370-2321.

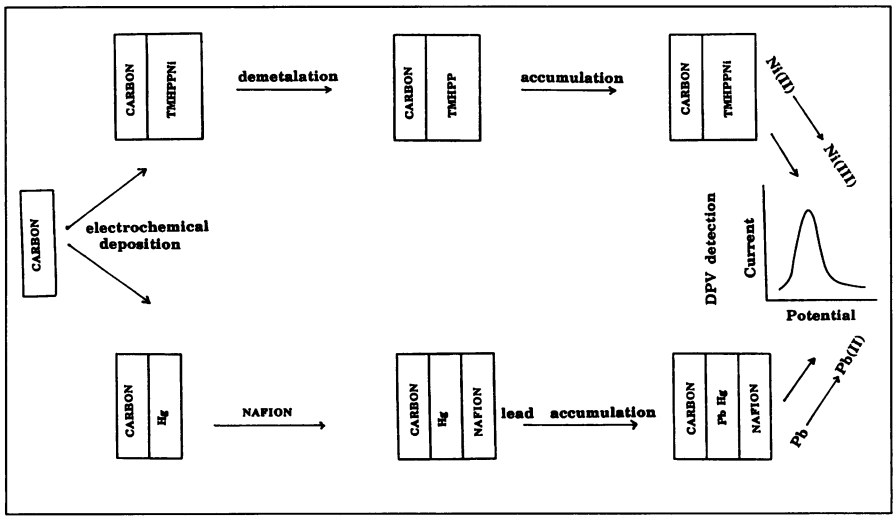

Figure 1. Steps involved in the determination of nickel by porphyrinic sensor (*a*) and the determination of lead by anodic stripping voltammetry (*b*) on carbon fiber electrode.

Microelectrode Sensor Fabrication and Modification

The microsensors were produced by threading a carbon fiber through a pulled end of a capillary with 1 cm of the fiber left protruding (*10*). The glass–fiber electrode interface was sealed with a nonconductive epoxy; a copper lead was inserted in the opposite end and sealed with silver epoxy cement. The single-fiber electrode was thermally sharpened using a propane microburner, coated with molten beeswax–rosin mixture, and sharpened again. The active surface had a length of 2 to 6 μm with a fiber diameter of 0.5 to 0.8 μm. The surface of the sharpened carbon fiber was modified to be selective and sensitive for Ni(II) or Pb(II).

The modification process for Ni(II) detection involves four steps: depositing poly-TMHPPNi film on the electrode surface, confirmation of deposition, demetalation of poly-TMHPP, and confirmation of demetalation.

Polymeric film is deposited from 5×10^{-4} M TMHPPNi solution in 0.1 M NaOH using constant-potential electrolysis at 0.7 V. At the end of the deposition time, the electrode is rinsed and immersed in 0.1 M sodium hydroxide. The presence of poly-TMHPPNi film on the carbon surface is confirmed by a peak at E_p= 0.55 V, attributed to the Ni(II)–Ni(III) couple in a DPV scan. The poly-TMHPPNi film is demetalated by placing the electrode in stirred 0.1 M HCl for 120 sec.

The absence of the Ni(II)–Ni(III) peak in a DPV scan between 0.0 and 0.8 V in 0.1 M NaOH confirms the absence of Ni(II) in the poly-TMHPP film. A detailed characterization of the poly-TMHPP electrode as a micro- and ultramicro-ion exchange sensor for nickel has been published previously (*11,12*).

The carbon fiber microelectrode for Pb(II) detection was plated with mercury film. A 9.9 ml of the acetate buffer solution and 0.1 ml of the 5×10^{-3} M mercury solution were introduced into the electrolytic cell. The solution was purged with argon for 5 min and mercury was deposited at constant potential of –0.8 V. When the coulometrically controlled mercury film thickness reached the desired value (0.02 to 0.05 μm), the potential was changed to –0.1 V, thereby removing metallic contamination. Finally, the electrode was covered with a thin film of cation exchanger by immersing the carbon fiber/Hg electrode in 1% nafion solution for 10 sec.

Determination of Total Concentration of Ni(II) and Pb(II)

Total bound and unbound nickel concentrations in cells were determined by liquid scintillation counting of ^{63}Ni according to the described procedure (*13*). In a parallel experiment, nickel and lead concentrations were determined by using inductively coupled plasma ICP emission spectroscopy (*3*). Wavelengths of 231.6 and 220.3 nm were used for detection of Ni and Pb, respectively. Model 1160 Plasma Atomcomp and ICP polymonochromator (Jarrel-Ash, Waltham, MA) were used.

Results and Discussion

Sensing Unbounded Metals in a Single Cell

Two designs of sensors for nickel and lead which can be applied to measurements in single cells are shown in Figure 1. The sensor based on poly-TMHPP is highly sensitive and selective for nickel. Initial oxidation of the monomeric, metalated TMHPP units leads to polymerization and formation of a highly conductive film on the surface of the carbon fiber (Figure 1*A*). The polymer undergoes facile demetalation in an acidic solution leaving on the electrode surface an intact, adherent, conductive film that may selectively incorporate Ni(II) cations from analyte solutions by an ion exchange process in which protons from the porphyrin ring are exchanged for Ni(II). Current from Ni(II)/Ni(III) oxidation generates the analytical signal, observed at 0.55 V. Because both Ni(II) and Ni(III) cations remain in the porphyrin film, the sensor can be transferred to a 0.1 M NaOH solution where oxidation of Ni(II) to Ni(III) is very efficient and produces a high current voltammetric analytical signal. At low nickel concentration, a catalytic current due to oxidation of water on poly-TMHPPNi film can be used as an analytical signal. The detection limit is 5×10^{-5} to 10^{-6}, M based on the Ni(II)–Ni(III) process and water oxidation process, respectively. No significant interferences due to the presence of Zn, Cd, Pb, Cu, Fe, and Co are observed.

The sensor for lead is based on electrochemical preconcentration of Pb(0) in the thin layer of mercury deposited electrochemically on a carbon fiber (Figure 1*B*). The deposition process is followed by electrochemical oxidation of Pb(0) to Pb(II). The stripping process, occurring at –0.48 V, produces a current proportional to the Pb concentration in the mercury film and bulk solution. Anodic stripping voltammetry, and especially differential-pulse stripping voltammetry, is a powerful

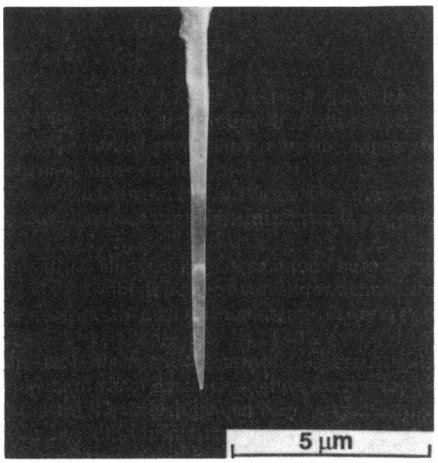

Figure 2. Tip of thermally pointed carbon fiber sensor covered with polymeric TMHPP.

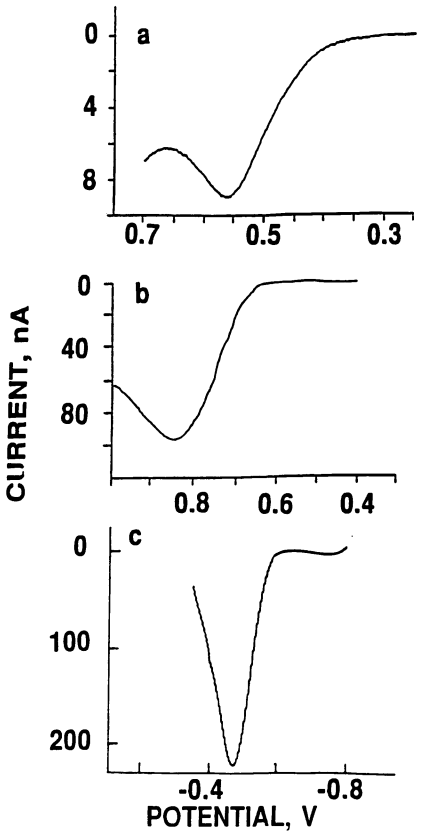

Figure 3. Differential-pulse voltammogram of nickel obtained with polymeric TMHPPNi film in 0.1 M NaOH; nickel was preconcentrated from 10^{-4}M solution (*a*). Differential-pulse voltammogram of catalytic oxidation of water obtained with polymeric TMHPPNi film in 0.1 M NaOH nickel was preconcentrated from 10^{-5} M solution at pH 7.4 for 15 min (*b*). Anodic stripping voltammogram of lead obtained in 0.1 M HCl; lead was preconcentrated in mercury/nafion film from 5×10^{-7} M solution at pH 7.4 for 10 min (*c*).

electroanalytical technique for trace metal measurements (*14*). The detection limit of this sensor is 5×10^{-8} M at pH 7.4; however, the Pb microsensor is not as selective as the previously described nickel sensor.

Optimization of the Carbon Fiber Microelectrode

Carbon fibers with diameters of 6 to 8 μm are sharpened to 0.2 to 0.8 μm in order to avoid cell membrane rupture during the implantation process. A carbon fiber was sharpened following a procedure described previously (*10*) using a microburner. Subsequently, the sharpened fiber was immersed in melted beeswax–rosin mixture kept at a controlled temperature for 5 to 15 sec. After cooling to room temperature, the electrode was resharpened. An electrode obtained by this procedure has several features important for single-cell

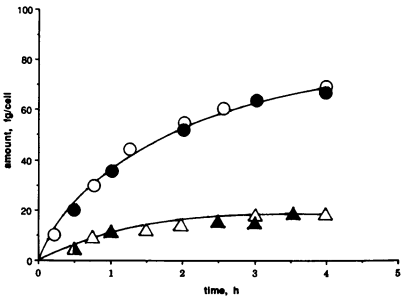

Figure 4. Total concentration of bound and unbound nickel in a BC3H-1 cell (○) and in a H4-II-C3 cell (△). Nickel was determined by the ^{63}Ni liquid scintillation method (○,△) and ETV-ICP method (●,▲). Cells were incubated in 10^{-4} M Ni(II) solution from 15 min to 4 hr.

applications, including a smaller diameter but longer sharpened tip. The resulting electrode is a slim cylinder with a small diameter rather than a short taper. The classical procedure for fabricating microelectrodes by inserting a carbon fiber into a glass microcapillary followed by polishing cannot be applied since the electrode dimensions are not appropriate. The diameter of the electrode includes the glass and the fiber, making it too large for insertion into single cells with diameters smaller than 10 μm. Since the electroactive area is limited to the circular disk of the exposed electrode tip, and because the current (analytical signal) is directly proportional to the surface area, it is important to make this area as large as possible for the smallest possible diameter. Since the ratio, R, of surface area of a cone-shaped electrode to that of a disk electrode is b/r (b is the slant height of the cone and r is the radius of the cone or disk) and typical electrode dimensions are r=0.25 μm and b=5 μm, the surface area of the cone-shaped electrode will be 20 times that of the disk electrode, so the resulting analytical signal will also be higher by this same factor. Generally, the electrode length should be less than one cell thick; however, electrodes can be implanted into flat cells from any convenient angle, making this restriction less critical.

Accumulation of Nickel in Cells

Chronological changes in the total nickel concentration accumulated by H4-II-C3 rat hepatoma cells and BC3H-1 myocytes are shown in Figure 4. Nickel was measured using liquid scintillation counting of ^{63}Ni and ICP spectroscopy with electrochemical preconcentration followed by electrothermal evaporation. After incubation for 1 hr, the total amount of bound and unbound nickel in the BC3H-1 cell and H4-II-C3 cell is 36 and 12 fg, respectively. After 4 hr of incubation this amount is almost doubled to 70 fg per BC3H-1 cell and 20 fg per H4-II-C3 cell. However, there is a significant difference between the accumulation of nickel between the two cell lines studied. The ratio of 3 in the accumulated nickel in the BC3H-1 cell relative to that in the H4-II-C3 cell remains constant throughout the incubation time. After 4 hr of accumulation, a concentration-time plot reached a plateau, and a significant increase (10 to 25%) in cell death is observed.

The amount of unbound nickel determined using the poly-TMHPPNi microsensor after 1 hr incubation was found to be 1 and 4 fg per single H4-II-C3 and BC3H-1 cell, respectively. These represent amounts about 10% of the total amount of nickel accumulated by these cells. A plot of the amount of unbound nickel per cell vs time is sigmoidal. The amount of unbound nickel increases slightly up to about 2 hr of incubation time. At this point, an inflection on the concentration-time curve is observed, followed by a rapid increase of Ni(II) concentration; finally between 3 and 3.5 hr, a plateau is reached. The amount of

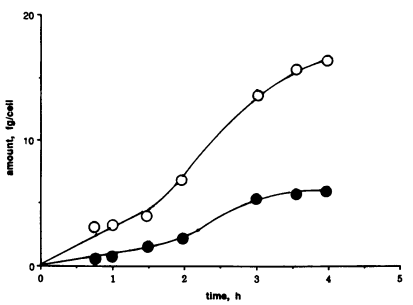

Figure 5. Unbound nickel concentration in a single BC3H-1 (○) and a single H4-II-C3 cell (●) determined by carbon fiber TMHPP sensor.

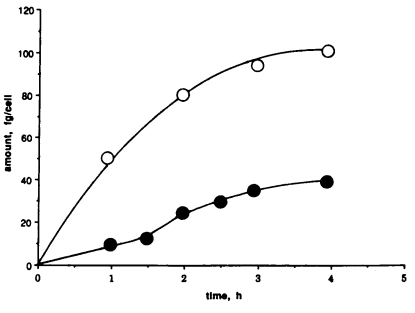

Figure 6. Total concentration of bound and unbound lead (○) and unbound lead (●) in a BC3H-1 cell. Cells were incubated in 10^{-4} M Pb(II) from 15 min to 4 hr.

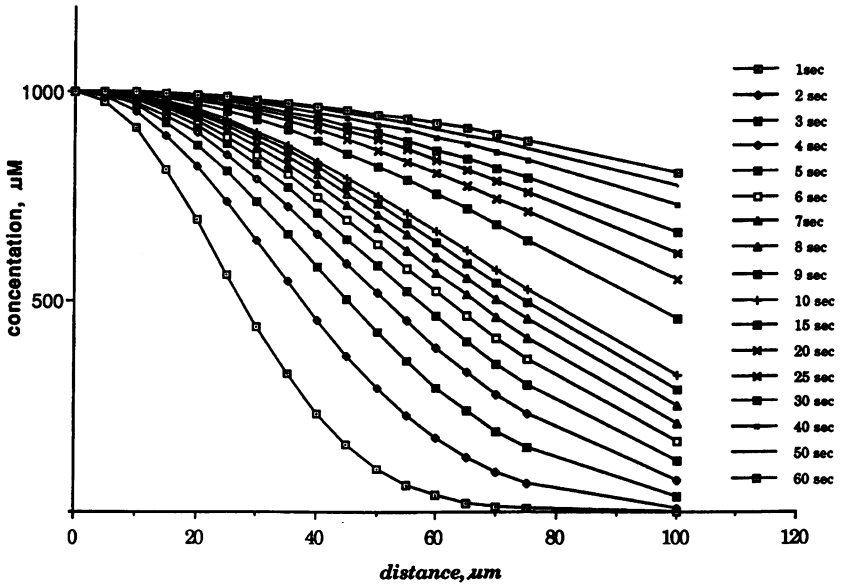

Figure 7. Simulation curves obtained based on Fick's equation for diffusion of nickel.

unbound Ni(II) after 4 hr of incubation is about 25 to 30% of the total nickel accumulated. Molar concentration of unbound nickel in both cells after 4 hr of incubation approaches 10^{-4}, i.e., the concentration of nickel in the incubation solution.

A pattern of accumulation of Pb(II) in BC3H-1 cells is very similar to that observed for nickel accumulation. Figure 6 shows the changes in the total amount of bound and unbound lead in BC3H-1 myocytes as a function of incubation time in 1×10^{-4} M lead. The total amount of lead after 1 hr incubation is 50 fg in a single cell, increasing to 80 fg after 2 hr incubation. After that, only a slight increase of the amount of lead concentration is observed. A plot of the amount of unbound lead vs time shows a sigmoidal shape with an inflection point between 1.5 and 2 hr. The molar concentration of lead in the cell approaches that of the incubation solution after 3 hr. A rabbit aorta ring was used for studies of diffusion of metals into the tissue.

Assuming that diffusion of cations in the tissue is not hindered by chemical bonding, the diffusion process should be described by Fick's equation, which for planar diffusion is (*15*):

$$\left(\frac{\partial_c}{\partial_x}\right)_{xt} = \frac{Co}{(4\pi Dt)^{1/2}}\exp\left(\frac{x^2}{4DT}\right) \qquad [1]$$

where Co is an initial concentration, c is the concentration of a distance x and time t, x is the distance from plane where $c = Co$, T is temperature and D is diffusion coefficient. Knowing the diffusion coefficients ($D = 1.331\times10^{-5}$ and 1.891×10^{-5} cm^2/sec for Ni(II) and Pb(II), respectively) and the initial concentration ($Co = 10^{-4}$ M), the expected concentration, c, of Ni(II) and Pb(II) can be estimated in tissue for a given time and distance. An example of these simulation curves for diffusion of Ni(II) is shown in Figure 7. Thus, after 15 sec of tissue incubation in 10^{-4} M solution, the concentration Ni(II) at a distance $x = 100$ µm should be about 50 µM. A ring of porcine aorta was filled with 10^{-4} M Ni(II) solution and sensors were implanted in muscle cells at different distances from the aorta inferior wall (composed of a layer of endothelial cells). After 60 sec no detectable concentration of nickel was found at the distance $x = 100$ µm. The nickel concentration of 50 µM was detected by the sensor after 2 hr of incubation, a result indicating that mass transport of nickel in the tissue is not diffusion controlled.

A similar effect is also observed for Pb. Several processes hinder free diffusion, and a cell can use several types of aqueous traps for ions: *a*) the cell membrane can be used as a physical barrier to diffusion; *b*) ions can be stored free or bound within an internal vesicle or bound to internal polymers; *c*) internal polymers can incorporate an ion forming unstable bonds; or *d*) two trapped ions can combine forming a precipitate (*1*). The data presented indicate that relatively high amounts of nickel as well as lead can accumulate in the types of cells studied. This result has been noted previously for nickel and attributed to the fact that the cell membrane is negatively charged, thus competing for nickel binding with other ligands. Nickel internalization by the cell is possible by either active transport, most likely through calcium and/or magnesium channels, or passive diffusion of neutral, lipophylic complexes (*16*) while the exact nature of the nickel binding sites in the cell is unknown. Studies have pointed to the involvement of phosphate groups of both RNA and, to a lesser extent, DNA, along with proteins, phospholipids, amino acids, mononucleotides, and other ligands. These extensive nickel-binding capabilities within the cell account for the relatively slow mass transport through the cell and the relatively small proportion of the total nickel left unbound. After saturation of all active sites with nickel, observed after about 2 hr of incubation, concentration of free nickel in the cell is approximately the same as in the incubation solution.

Lead can bind in many of the same sites as K^+ and can replace Ca^{2+} with loss of functional and structural integrity. Lead can also replace Zn^{2+} in 5-aminolavulinic acid dehydratase and reacts much faster than Ni(II) with sulfydryl groups. The present data clearly indicate that most of the nickel and lead is bound to biological material. Presumably, after longer accumulation time when all nickel- and lead binding sites become saturated, the gradient concentration of free nickel and lead between the cell and the incubation solution should be zero. However, the cells under study were damaged by the toxic effects of the accumulated nickel, especially accumulated lead. A significant number of cells, particularly the BC3H-1 myocytes, died after an incubation of more than 4 hr.

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